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ORIGINAL ARTICLE

Application of high performance thin-layer chromatography to separation of oleanolic, ursolic and betulinic acids

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Abstract: High performance thin-layer chromatography on silica was used for the separation of three triterpenic acids. Because of the similarity of chemical structures of oleanolic and ursolic acid, isocratic chromatography using a mixture of two or three solvents was not successful; however, the multiple gradient development (MGD) technique was more suitable. Oleanolic and ursolic acid were separated after five steps of development with mixtures of petroleum ether/ethyl acetate in concentrations 20%,18% and 15%. The mixture of cyclohexane/ethyl acetate/formic acid (7.5:2.5:0.05 v/v) was used in the last step to improve the shape of the chromatographic bands. The progress of separation was controlled after each step of development by use of densitometer Desaga CD 60. The measurements were carried out after derivatization of 10% H_2SO_4 in ethanol at λ = 515 nm in absorbance mode.

Key words: high performance thin-layer chromatography (HPTLC), oleanolic acid, ursolic acid, betulinic acid, multiple gradient development

INTRODUCTION

Oleanolic, ursolic and betulinic acids belong to pentacyclic triterpenes and are common constituents of many medicinal herbs and plants [1]. These terpenes may exist in the form of free acids or as aglycones for triterpenoid saponins. They have many important pharmacological effects. In the literature there are numerous data on their anti-inflammatory, hepatoprotective, anti-tumour, anti-HIV, anti-microbial, anti-fungal, anti-ulcer, gastroprotective, hypoglycemic and anti-hyperlipidemic properties [2-8]. They are relatively non-toxic and have been used in cosmetics and health products, e.g. oleanolic acid is marketed in China as an oral drug for human liver disorders, and ursolic acid is used in anti-tumour therapy in Korean traditional medicine.

The most explored method in the determination of these compounds is HPLC [9-14] but TLC is still an important tool of phytochemical investigations. Its advantages are the low amount of organic solvent used in the separation process and possibility of application of samples without any pretreatment.

These triperpenes, especially oleanolic and ursolic acids, have a similar molecular structure (Fig. 1) which makes their separation by thin-layer chromatography very difficult.

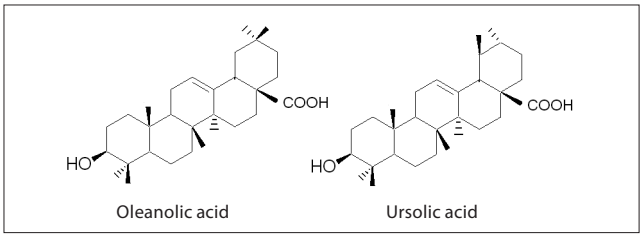

Figure 1 Structures of investigated compounds.

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This paper presents results of the application of HPTLC to the separation of oleanolic, ursolic and betulinic acids on silica gel.

MATERIALS AND METHODS

All solvents were pro analysis grade from Polish Reagents (POCh, Gliwice, Poland).

Triterpenic acids standards of the highest grade were purchased from Sigma (St. Louis, MO, USA). All samples were prepared as 0.05 % solutions in methanol.

Extracts of *folium Salviae* (*Salvia officinalis* L.), *folium Plantaginis lanceolatae* (*Plantago lanceolata* L.) and *flos Lamii albi* (*Lamium album* L.) were prepared by 24 h extraction with diethyl ether at room temperature. The ether extracts were evaporated to dryness and the residues were dissolved in 2 mL of acetone.

In the experiments, HPTLC plates 10 × 10 cm coated with silica gel (Merck, Darmstadt, Germany) were used. The plates were washed with methanol and dried in a stream of hot air before use.

2 μL of standard solutions and extracts were spotted using an automatic applicator Desaga AS 30 (Heidelberg, Germany) under nitrogen at 2.5 atm as streaks 6 mm long.

Chromatograms were developed in horizontal Teflon DS chambers (Chromdes, Lublin, Poland).

To separate the mixtures of oleanolic, ursolic and betulinic acid multiple development was used according to the programme shown in Table 1. The mobile phase was removed by evaporating in a stream of warm and then cold air for 10 min after each step of development.

The plates were sprayed with 10% m/m H_2SO_4 in ethanol, dried, and then heated to 110^0 C for 2-3 min. After derivatization the chromatograms were observed in daylight or in UV light at λ = 366 nm. The densitograms were obtained using Desaga CD-60 densitometer (Heidelberg, Germany) controlled by a Pentium computer in absorbance/reflectance mode at λ = 515 nm.

Documentation was obtained in daylight with use of a digital camera.

RESULTS AND DISCUSSION

Triterpenic acids are an important group of natural compounds with confirmed pharmacological activity. They occur simultaneously in many medicinal herbs and plants.

The closeness of their chemical structures, especially oleanolic and ursolic acids, makes their TLC separation very difficult. There are some chromatographic systems for analysing triterpenes, given in the literature [15-18] but none of them enable the separation of oleanolic and ursolic acids.

To find the most suitable eluent, numerous tests using various organic solvents were performed. The mixtures of weakly polar solvents: heptane, cyclohexane, petroleum ether, toluene, dichloromethane with acetone, diisopropyl ether, diethyl ether, ethyl acetate, methanol, 2-propanol, butanol in various ratios were tested.

In most investigated chromatographic systems betulinic acid was well separated but hR_F values for oleanolic and ursolic acids were identical. Small differences of hR_F values were observed in mixtures of cyclohexane/ethyl acetate, petroleum ether/diisopropyl ether and petroleum ether/ethyl acetate, but the separation of the mixture of oleanolic and ursolic acids was poor.

In several publications, Matysik et al. demonstrated that the multiple gradient development technique (MGD) is useful for separating compounds with similar chemical structures [19,20]. MGD technique requires evaporation of the mobile phase from the plate before the next step of development; therefore, due to their volatility, the mixtures of petroleum ether/diisopropyl ether and petroleum ether/ ethyl acetate were chosen for further investigations.

To optimize the gradient programme of chromatogram development the relationships between hR_f values and mobile phase composition were determined. The differences of hR_F values were observed in the range of concentrations 40-60% of diisopropyl ether and 15-20% of ethyl acetate in petroleum ether. The example of plots of hR_F against % of modifier are presented in Figure 2.

The best result was obtained using the gradient programme shown in Table 1. The presented chromatographic system is

Table 1 Programme of gradient elution

Step No.	Solvents	Distance of development (cm)	Time of development (min.)
1	Petroleum ether/ethyl acetate (8:2 v/v)	7	15
2	Petroleum ether/ethyl acetate (1.8:7.2 v/v)	8	15
3	Petroleum ether/ethyl acetate (1.5/8.5 v/v)	9	17
4	Petroleum ether/ethyl acetate (1.5/8.5 v/v)	9	17
5	Cyclohexane/ethyl acetate/HCOOH (2:8:0.05 v/v)	9	25

After each step, the plate was dried for 10 min in a stream of warm and then cold air

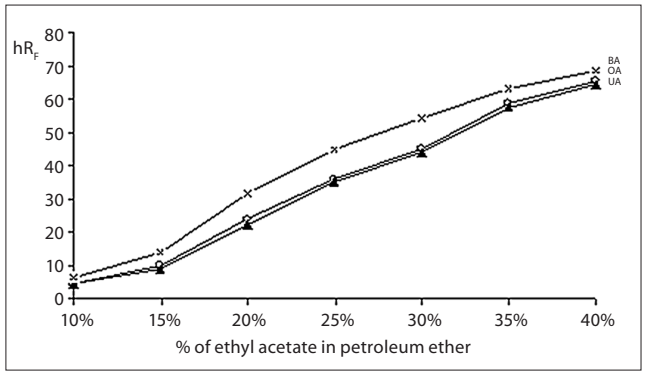

Figure 2 Relationships between hR_F values and concentration of ethyl acetate in petroleum ether; OA – oleanolic acid, UA – ursolic acid, BA – betulinic acid.

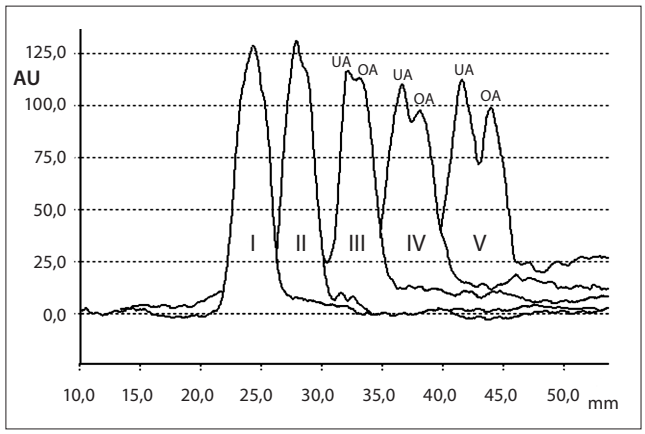

Figure 3 Densitogram of mixture of oleanolic acid (OA) and ursolic acid (UA) obtained after each step of development. Measurements were carried out at λ=515 nm after derivatisation of 10 % (v/v) methanol H_2SO_4.

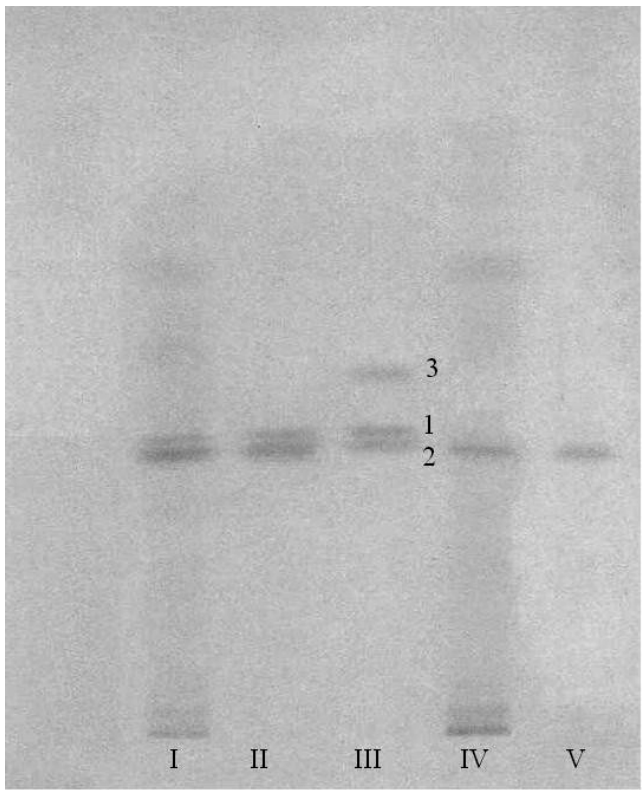

Figure 4 Chromatogram of herbal extract obtained using gradient programme according Table I. I – extract of *folium Plantaginis lanceolatae*; II – extract of *folium Salviae;* III mixture of standards (1 – oleanolic acid, 2 – ursolic acid, 3 – betulinic acid); IV – *flos Lamii albi*; V – standard of ursolic acid. Documentation was obtained after derivatisation of 10 % (v/v) methanol H_2SO_4 in daylight.

not suitable for quantitative analysis of oleanolic and ursolic acids because the resolution of peaks is insufficient (Fig. 3) but enables their identification in plant materials. The presented methods were successfully applied to the identification of triterpenic acids in extracts of *Salvia officinalis herba, Lamium album flos, Plantaginis lanceolatae folium* (Fig. 4).

CONCLUSION

The similarity of chemical structures of triterpenic acids makes their separation on silica very difficult. Isocratic planar chromatography was not successful, however, the MGD technique permitted satisfactory separation of all investigated compounds after five steps of development using mixtures of ethyl acetate/ petroleum ether (steps 1-4) and a mixture of ethyl acetate/cyclohexane/formic acid 2.5:7.5:0.5 v/v (step 5).

The presented gradient programme can be used for the identification of oleanolic, ursolic and betulinic acids in plant material.

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REFERENCES

1. Janicsák G, Veres K, Kakasy AZ, Máthé I: Study of the oleanolic and ursolic acid contents of some species of the *Lamiaceae Biochem Syst Ecol* 2006, **34**, 392-396.
2. Liu J: Pharmacology of oleanolic acid and ursolic acid. *J Ethnopharm* 1995, **49**, 57-68.
3. Liu J: Research perspectives. *J Ethnopharm* 2005, **100**, 92-94.
4. Ma Ch, Cai S, Cui J, Wang R, Tu P, Hattori M, Daneshtalab M: The cytotoxic activity of ursolic acid derivatives. *Eur J Med Chem* 2005, **40,** 582-589.
5. Baglin L, Mitaine-Offer AC, Nour M, TanK, Cavé C, Lacaille-Dubois MA: A review of natural and modified betulinic, ursolic and echinocystic acid derivatives as potential antitumor and anti-HIV agents. *Mini Rev Med Chem* 2003, **3,** 159 -161.
6. Gbaguidi F, Accrombessi G,. Moudachirou M, Quetin-Leclercq J: HPLC quantification of two isomeric triterpenic acids isolated from *Mitracarpus scaber* and antimicrobial activity on *Dermatophilus congolensis. Pharm Biomed Anal* 2005, **39**, 990-995.
7. Farina C, Pinza M, Pifferi G: Synthesis and anti-ulcer activity of new derivatives of glycyrrhetic, oleanolic and ursolic acids. *Farmaco* 1998, **53**, 22-32.
8. Paduch R, Kandefer-Szerszeń M, Trytek M, Fiedurek J: Substances useful in human healthcare. *Arch Immunol Ther Exp* 2007, **55,** 315-327.
9. Song M, Hang T, Wang Y, Jiang L, Wu X, Zhang Z, Shen , Zhang Y: Determination of oleanolic acid in human plasma and study of its pharmacokinetics in Chinese healthy male volunteers by HPLC tandem mass spectrometry. *J Pharm Biomed Anal* 2006, **40**, 190-196.
10. Cen JH, Xia RX: High-performance liquid chromatographic analysis of bioactive triterpenes in *Perilla frutescens***.** *J Pharm Biomed Anal* 2003, **32**, 1175-1179.
11. Büchele B, Zugmaier W, Simmet T: Analysis of pentacyclic triterpenic acids from frankincense gum resins and related phytopharmaceuticals by high-performance liquid chromatography. Identification of lupeolic acid, a novel pentacyclic triterpene. *J Chromatogr B* 2003**, 791**, 21-30.
12. Claude B, Morin PH, Lafosse M, Andre P: Evaluation of apparent formation constants of pentacyclic triterpene acids complexes with derivatized β- and γ-cyclodextrins by reversed phase liquid chromatography. *J Chromatogr A* 2004, **1094**, 37-42.
13. Monte FJ, Kintzinger JP, Trendel JM, Poinsot J: Mixture of closely related isomeric triterpenoid derivatives: Separation and purification by reversed-phase high-performance liquid chromatography. *Chromatographia* 1997**, 46,** 251-255.
14. Liao LP, Li SL, Li P: Simultaneous determination of seven triterpenoids and triterpenoid saponins in *Folium Ilicis Purpureae* by high performance liquid chromatography coupled with evaporative light scattering detection. *J Sep Sci* 2005**, 28**, 2061-2066.
15. Banerjee A, Sane RT, Mangaonkar K, Shailajan S, Deshpande A, Gundi G: Quantitation of oleanolic acid in *Oldenlandia corymbosa* L. whole-plant powder by high-performance thin-layer chromatography. *J Planar Chromatogr* 2006, **19,** 68-72.
16. Baricevic D, Sosa S, Della Loggia R, Tubaro A, Simonovska B, Krasna A, Zupancic A: Topical anti-inflammatory activity of *Salvia officinalis* L. leaves: the relevance of ursolic acid. *J Ethnopharmacol* 2001, **75**, 125-132.
17. Wagner H, Bladt S: *Plant Drug Analysis*, Springer-Verlag, Berlin/ Heidelberg/New York 1996, 305-326.
18. Shetty P, Mangaonkar K, Sane RT: HPTLC determination of ursolic acid in *Alstonia scholaris. J Planar Chromatogr* 2007, **20**, 65-68.
19. Matysik G: Modified programmed multiple gradient development (MGD) in the analysis of complex plant extracts. *Chromatographia* 1996, **43,** 39-43.
20. Pobłocka L, Matysik G, Lisowski W: Multiple gradient TLC and densitometric analysis of p-coumaric acid in some medicinal plants from the family *Solanaceae. J Planar Chromatogr* 2003, **16,** 76-79.